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Fibroblast growth factor binding protein (FGF-BP) releases immobilized FGF's form the extracellular matrix and can function as an angiogenic switch molecule in cancer. We have determined that FGF-BP is upregulated in a portion of breast cancer and this upregulation is correlated with increased expression of beta-catenin. In this grant, we hypothesized that beta-cantenin can initiate angiogenesis in mammary carcinoma through FGF-BP. The aims were 1) to study the expression of FGF-BP in mammary tumorigenesis progression of the APC/+ mouse and 2) to determine the mechanism of regulation of FGF-BP by the APC/beta-catenin signaling pathway in breast cancer. To date, we have shown a positive correlation of upregulation of beta-catenin expression and FGF-BP in breast and other tumors in the APC/+ mice. We have also shown that beta-catenin can directly induce FGF-BP gene expression through a transcriptional mechanism and that a TCF site in the FGF-BP promoter is responsible for a major portion of this effect.

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INTRODUCTION

Breast cancer is dependent on angiogenesis for growth and malignant progression. Without angiogenesis, the tumors would not have the proper nutrients for growth and would be limited in their ability to enter the circulatory system and metastasize [1]. The process of angiogenesis is controlled and regulated by a number of different protein factors. FGF-2 is one of the most potent of the pro-angiogenic factors [2]. FGF-2 is normally sequestered in the extracellular matrix (ECM) and can be released by enzymatic cleavage of heparin sulfate proteoglycans or by the fibroblast growth factor binding protein (FGF-BP) [3]. FGF-BP can reversibly bind to FGF-2 and release it from the ECM [3]. Previous work from our laboratory indicates that FGF-BP acts as an angiogenic factor and is expressed in a subset of breast cancers as well as in squamous cell carcinoma and colon carcinoma as indicated by histological analysis of human tumor samples (Ray et al appendix manuscript). We examined the expression of FGF-BP in the mammary tumors of the Min/+ mouse. This mouse model has a germline mutation in the adenomatous polyposis coli (APC) gene. A second somatic hit in the second allele of this gene produces a non-functional APC protein [4]. This mutation results in overexpression of the oncogene beta-catenin. In this study we examine the regulation of FGF-BP1 by beta-catenin in breast cancer.

BODY

We have previously shown that FGF-BP is expressed in the mammary tumors of the Min/+ mouse. In this report and the previous report work was accomplished toward the goals stated in Aim 2 (To study the mechanism of regulation of FGF-BP by the APC/beta-catenin signaling pathway in breast cancer) are discussed. First, it was necessary to confirm that FGF-

BP was indeed regulated by beta-catenin. Although FGF-BP was coexpressed with beta-catenin in mammary tumors of the Min/+ mouse, this only confirms spatial correlation of the two proteins. In order to establish a direct relationship between beta-catenin and FGF-BP, a series of *in vitro* assays were undertaken using two different breast cancer cell lines. The MDA-MB-468 breast cancer cell line expresses endogenous FGF-BP and low levels of beta-catenin. This cell line was used to test whether increases in beta-catenin levels would effect the endogenous expression of FGF-BP. To induce higher levels of beta-catenin, the MDA-MB-468 cells were treated with lithium chloride. Lithium inhibits glycogen synthase kinase-3beta (GSK-3beta) a negative regulator of beta-catenin. Upon treatment of MDA-MB-468 with Lithium chloride, which resulted in a subsequent increase of cytoplasmic and nuclear beta-catenin, there was a 3-fold induction of FGF-BP1 mRNA levels (appendix manuscript). **Therefore, beta-catenin can regulate the endogenous FGF-BP1 gene product in breast cancer cell lines.**

As stated in Aim 2- experimental series #2, the possibility that beta-catenin regulates FGF-BP at the transcriptional level has been examined. To determine whether FGF-BP regulation by beta-catenin occurs at the promoter, we transiently co-transfected the SK-BR-3 breast cancer cell line with a 1Kb portion of the FGF-BP promoter (-1060/+62 FGF-BP-luciferase) and a wild-type beta-catenin expression vector (appendix manuscript). We found that beta-catenin is able to activate the promoter up to 3.5 fold in breast cancer cells (appendix manuscript). Furthermore, E-cadherin, which sequesters beta-catenin, was co-transfected with FGF-BP and beta-catenin into breast cancer cells and was able to reverse the beta-catenin-mediated induction of FGF-BP (appendix manuscript). These results verify that FGF-BP is a direct target of beta-catenin and that it occurs at a transcriptional level. Many beta-catenin target genes are transcriptionally regulated via a TCF-site (5'-A/T A/T CAAAG-3') [5]. In order to

determine the relevant promoter regions of FGF-BP for beta-catenin regulation, a series of FGF-BP promoter fragments were cloned into the PGL3 empty vector. These fragments were sequential 5'-deletions of the promoter beginning from a 1 Kb piece. Some of these constructs were created using restriction enzyme digestions while the others were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). These progressive 5' deletion constructs of FGF-BP +/- beta-catenin were transiently transfected into the SK-BR-3 breast cancer cell line (Fig 4 in appendix manuscript). Deletion of the region –1060 to –118 caused a loss of about 60% of the inductive effect and further deletion of –93 to –77 caused complete loss of the remaining induction (Fig 4 in appendix manuscript). In the upstream region a large portion of the beta-catenin inductive effect is mediated by a TCF site at –1030 (Fig4 in appendix manuscript). We are currently determining what other factors are involved in beta-catenin upstream regulation of FGF-BP and also what factors are responsible for regulation between –93 and –77. In the last report we had preliminary data that the FGF-BP promoter might be regulated by a non-TCF site that was located between –152 and -139 of the FGF-BP gene promoter. However, in repeat experiments deletion of this site did not give a consistent abrogation of the beta-catenin inductive effect. To underscore the importance of beta-catenin to FGF-BP gene promoter regulation we determined that in cells where the beta-catenin allele is knocked out, we see substantially less promoter activity than in the wild type cells (Fig 4 in appendix manuscript)

Key Research Accomplishments

- Identification of a relevant genetically defined mouse model of breast cancer to study the expression pattern and function of the angiogenic factor FGF-BP.
    - We have identified FGF-BP as a novel target gene of the WNT/beta-catenin signaling pathway.
    - We have identified a TCF site in the FGF-BP gene promoter that explains a significant portion of the beta-catenin inductive effect. In addition more proximal regions of the gene promoter are also involved in regulation and are currently studies are done for their role in the beta-catenin induction of FGF-BP in breast cancer.
    - We have shown that loss of a beta-catenin allele causes substantial reduction in FGF-BP gene promoter activity.

Abstracts

Ray, R., Cabal-Manzano, R., Riegel, AT., Wellstein, A. The Angiogenic Factor Fibroblast Growth Factor Binding Protein (FGF-BP), a novel beta-catenin target gene. American Association of Cancer Research, New Orleans, LA (2001).

Papers: **Ray, R** Cabal-Manzano-R, Moser A.R., Waldmann,T., Zipper,LM, Aigner,A, Byers,S.W. Riegel,A.T. and Wellstein A. Upregulation of FGF-BP by beta-catenin during colon carcinogenesis. Cancer Research In Press (appendix manuscript) (this paper contains all the regulatory data in the breast cancer cell lines showing that FGF-BP is regulated by beta-catenin (Fig 2-4)in a similar fashion to its upregulation by beta-catenin in colon cancer.

Conclusions

The goal of **Aim 2, experimental series #2** was to determine if FGF-BP1 is a target gene of the WNT/beta-catenin signaling pathway in breast cancer. Using a variety of *in vitro* techniques (transient transfections, northern blot analysis, western blot analysis, site-directed mutagenesis, etc.) we have confirmed that beta-catenin does regulate FGF-BP in breast cancer cell lines as well as other tumor types. Because beta-catenin is overexpressed in human breast cancer [6] it may possibly act as an oncogene in this disease as it does in colon cancer. Our work presents the possibility that beta-catenin may influence tumor angiogenesis in breast cancer through FGF-BP. In the past year we have refined our analysis of the FGF-BP gene promoter and determined that a TCF site at –1030 contributes a major portion of the beta-catenin effect. In addition more proximal regions of the promoter are highly involved in beta catenin regulation and these sites as well as the regulation of the TCF sites are currently under investigation in the laboratory.

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Upregulation of Fibroblast Growth Factor-Binding Protein by β-catenin during colon carcinogenesis.

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Abstract

Fibroblast Growth Factor-Binding Protein (FGF-BP) releases immobilized FGFs from the extracellular matrix and can function as an angiogenic switch molecule in cancer. Here we show that FGF-BP is upregulated in early dysplastic lesions of the human colon that are typically associated with a loss of APC (adenomatous polyposis coli) and upregulation of β-catenin. In addition, FGF-BP expression is induced in dysplastic lesions in ApcMin/+ mice in parallel with the upregulation of β-catenin. Also, in cell culture studies FGF-BP is induced by β-catenin through direct activation of the FGF-BP gene promoter. We conclude that FGF-BP is a target gene of β-catenin. (100 words)

Introduction

We have previously shown that the secreted binding protein FGF-BP can act as a chaperone for locally stored FGFs and enhance their angiogenic activity, thus allowing FGF-BP to serve as an angiogenic switch molecule in cancer (1). Consistent with a role for FGF-BP in cancer, ribozyme-targeted depletion of FGF-BP from human colon cancer or squamous cell carcinoma cells showed a rate-limiting role for FGF-BP in tumor growth and angiogenesis (1). Hence, we proposed that this molecule is one of the "angiogenic switch" mechanisms required for malignant progression (1-3).

We found that FGF-BP is expressed at high levels in the murine gut during embryonic development, downregulated in the adult (4, 5), but expressed at high levels in some colon cancer tissues and cell lines (1). To evaluate regulation of FGF-BP during colon carcinogenesis we initiated a series of studies with normal and pathological colon biopsies to determine at what stage of transformation the gene is upregulated. Here we report that FGF-BP expression is highly upregulated in dysplastic lesions, i.e. early on during colon carcinogenesis. These early lesions are associated with mutations in β–catenin and/or a loss of function of the APC tumor suppressor gene has been identified in over 80% of sporadic colon carcinomas (6). To assess the possible contribution of the loss of APC to FGF-BP upregulation, we used a well-defined murine model, the B6 ApcMin/+ mouse, which carries one mutant APC allele and develops polyps upon loss of the residual wild-type APC allele (7, 8). In this model we found that FGF-BP and β-catenin expression was induced in polyps, as well as in a rare aberrant crypt focus, the earliest discernible stages of transformation (9). Furthermore, cell culture studies show that increases in endogenous β-catenin by treatment with LiCl result in a significant

increase in FGF-BP mRNA levels and co-transfection assays demonstrate transcriptional activation of the FGF-BP gene promoter by β-catenin through TCF-sites. We conclude that FGF-BP is a novel target gene of the Wnt/β-catenin pathway.

Materials and Methods

Tissue samples, immunohistochemistry and in situ hybridization

Paraffin-embedded archival tissues were provided by the tumor tissue core facility of the Lombardi Cancer Center with patient identifiers removed. Dr. Moser (U. Wisconsin) and Drs. Herfarth and Schoelmerich (U. Regensburg) provided samples from mouse models. H&E stains of serial sections were reviewed by a pathologist to verify the diagnosis. The categorization followed Duke's classification (10). Serial sections of 4 μm were used for FGF-BP protein staining or FGF-BP mRNA detection by in situ hybridization. The in situ hybridization protocol was described earlier using human and mouse FGF-BP riboprobes (4, 11) that were digoxigenin-labeled using the DIG RNA labeling mix (Roche). For the immunohistochemistry a rabbit polyclonal anti-FGF-BP antibody (diluted 1:150 in 2% BSA/PBS) was used. As described earlier, this antibody recognizes murine, rat and human FGF-BP in archival tissue sections (5, 11). A β-catenin monoclonal antibody was purchased from Transduction Laboratories, Lexington, KY. Stained cells were divided into three grade levels: 0, negative (absence of color); grade 1, moderately stained with an obvious brown color; and grade 2, vividly stained stained dark brown. A tissue section was considered as negative when less than 30% of the same morphological structure (normal mucosa, dysplasia or cancer) showed any color (grade 0) (12). All others were scored as positive in the analysis. In a subset of samples angiogenesis was assessed after CD31 staining using light microscopy at 200x in areas containing the highest number of capillaries or hot spots as described earlier (13).

Cell culture, transfections and reporter assays

The cell lines CaCo-2 (colon cancer), SKBR3 and MDA-MB468 (breast cancer) were from the American Type Culture Collection (Springfield VA). The HCT-116 cell line with somatic cell knockout of the activated β-catenin allele were provided by Dr. Todd Waldman, Georgetown University (14). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS at 37°C and 5% CO_2. 24 hours before transfection, cells were seeded in 12-well plates at a density of $1x10^5$ cells/well in DMEM + 10% FBS. CaCo-2 cells: For each well 0.75 μg of DNA constructs and 10 μl of LipofectAMINE reagent were combined with 200 μl of OPTI-MEM1 (Life Technologies) and incubated for 30 minutes at room temperature. Appropriate amounts of OPTI-MEM1 were added to the solution to bring the volume to 1 ml and the mixture was placed on cells for 3 hours at 37°C. The cells were then washed two times with IMEM (Life Technologies) and incubated in DMEM + 10% FBS for 18 hours. SKBR3 cells: DNA constructs were mixed in a 1:2 ratio with FuGENE (Roche) reagent in serum-free IMEM and incubated at room temperature for 30 minutes. The FuGENE/DNA solution was then added to the cells with DMEM + 10% FBS media and incubated at 37°C for 18 hours. Transfection efficiency was determined by co-transfection with 4.0 ng of the *Renilla* luciferase reporter vector pRL-CMV (Promega). After the 18 hour incubation, cells were lysed in 100 μl of Passive Lysis buffer (Promega). 10 μl of the cell extract was assayed for firefly and *Renilla* luciferase activity with the Dual-Luciferase™ Reporter assay system (Promega). FGF-BP promoter constructs (-1,060/+62, -118/+62, -93/+62, -77/+62) were cloned into the pGL3 vector as previously described (15). Two consensus TCF sites (5'-A/T A/T CAAAG-3') located at -1030 and at -545 were deleted

by PCR based mutagenesis using the following primers: forward primers 1030-del-F: 5'-CAA ATG TCT GTT TAT ACA ACT TAA GAC CC-3' and 545-del-F: 5'-CAG TCA CCC ATT CAT TTA TTG AGA GTG G-3'; reverse primers 1030-del-R: 5'-GGG TCT TAA GTT GTA TAA ACA GAC ATT TG-3' and 545-del-R 5-CCA CTC TCA ATA AAT GAA TGG GTG ACT G-3'. All constructs were sequenced to confirm mutations. SKBR3 cells were transfected with 100 ng of luciferase cDNA, 0.1ng Renilla and 300 ng of pCDNA3 or 300 ng pCDNA3-β-catenin using Fugene 6 transfection reagent (Roche). Experiments were typically performed in duplicate and repeated as indicated in the legends to the figures. β-catenin, E-cadherin, and Topflash expression constructs were described previously (16). The pcDNA3 cloning vector was purchased from Invitrogen.

Northern analysis

MDA-MB468 cells were plated in 10 cm dishes and grown to 70% confluence in IMEM+10%FBS 24 hours before treatment. The cells were treated with LiCl (30 mM) + inositol or with NaCl (30 mM) + inositol dissolved in 10 ml of IMEM+10% FBS. 16 hours after initiation of treatment total RNA was isolated using the RNA STAT-60 protocol (RNA STAT-60™, Tel-Test, Friendswood, TX). 30 μg of total RNA were run on a 1.2% formaldehyde-agarose gel. Blotting and hybridization with the human FGF-BP probe were performed as previously described (15).

Data Analysis

The Prism/Graphpad program was used for data analysis. P-values <0.05 were considered significant.

Results

FGF-BP is upregulated during the initiation of human colon carcinogenesis.

We had previously observed that FGF-BP is highly expressed during murine gut development and is down-regulated in the adult mouse (4). FGF-BP is also highly expressed in some human colon cancer cell lines and tissues (1). The stages of malignant progression towards colon cancer have been well delineated and we hypothesized that analysis of colon biopsies of different pathologic stages could indicate possible genetic alterations associated with the upregulation of FGF-BP. A survey of human colon biopsy material (Fig. 1) showed that FGF-BP expression was detectable in 12 of 76 histologically normal appearing samples. In contrast with this, a high portion of the samples with moderate to severe dysplasia expressed FGF-BP (62 of 85; $p<0.0001$ normal versus dysplastic mucosa; chi-square test). Interestingly, even individual dysplastic crypts (closed arrow in Fig. 1A) located within otherwise normal mucosa (open arrow in Fig. 1) show expression of FGF-BP. It is of note that the increased expression of FGF-BP in dysplastic lesions coincides with a significant increase in blood vessel density from 80 $\pm$7 to 154 $\pm$9 vessels/field as measured by CD31 staining in the lamina propria (normal mucosa vs. severe dysplasia; $p<0.001$, t-test).

To assess whether FGF-BP would be upregulated by all pathologic alterations in the gut, we studied a series of biopsy samples from different stages of inflammatory bowel disease. Only 3 of 26 samples from patients with ulcerative colitis (n=18) or Crohn's disease without apparent dysplasia (n=8) showed detectable FGF-BP expression. This frequency of expression was not different from the normal mucosa controls ($p>0.05$, chi-square). Since inflammatory bowel disease does not induce FGF-BP expression, we

hypothesized that the upregulation of FGF-BP at the onset of colon epithelial malignant transformation may be due to an early genetic event, such as loss of the APC tumor suppressor function associated with the initiation of dysplasia. To address this question, we used the APC heterozygous B6 ApcMin/+ mouse (17) as an animal model system.

FGF-BP expression in B6 ApcMin/+ mouse adenoma coincides with cellular relocation and increase in β-catenin protein.

As a first step, we compared FGF-BP expression in the intestines of wild-type C57BL/6J mice relative to that in B6 ApcMin/+ mice. We found no differences in baseline expression of FGF-BP (not shown). This suggests that the loss of function of one allele in the B6 ApcMin/+ mice is not sufficient to alter the signal towards FGF-BP expression. In the normal epithelium β-catenin is sequestered at the membrane and is rarely found in the cytoplasm or nucleus (18) (see Fig. 2J). When APC becomes defective in intestinal crypt cells of B6 ApcMin/+ mice, regulation of β-catenin is lost (17) and the epithelium progresses to early stages of malignancy. We used the accumulation of cytoplasmic and nuclear β-catenin in microscopic sections as a read-out for the loss of APC function and probed serial sections of normal and adenoma tissues for both FGF-BP and β-catenin expression.

Tissues were harvested from animals between 91 and 132 days of age and analyzed for β-catenin protein by immunohistochemistry and for FGF-BP mRNA by *in situ* hybridization. Dysplastic lesions within normal mucosal tissue (Fig. 2A and E; darker H&E staining) showed an elevation of the β-catenin protein (Fig. 2B and F; brown stain) and of FGF-BP mRNA (Fig. 2C and G; dark blue stain). Fig. 2D and H show

negative controls for FGF-BP detection. In a survey of tissues we found that 21 of 27 adenomas were strongly positive for FGF-BP (>30% of the adenoma surface area; Fig. 2C and G). Adjacent normal intestine was also examined for FGF-BP expression, and only 5 of 19 samples showed any expression of FGF-BP ($p<0.001$, normal versus adenomas; chi-square, Fig. 2K). Upon careful inspection of serial sections, we found a striking coincidence of expression of FGF-BP mRNA (Fig. 2C and G) and of β-catenin protein (Fig. 2B and F) in the same tissue areas. We also searched tissues for the earliest microscopically discernible stage of dysplasia, aberrant crypt foci (ACF), that are observed at a rate of 0.14 per mouse (19). Although we observed only a single ACF, there was coincidence of expression of the two genes in cells that formed this ACF in the midst of normal mucosal areas (Fig. 2I and J).

In addition to the tissues derived from the ApcMin/+ model, we also examined sections from intestinal polyps in a dextran sulfate-induced model of inflammatory colon disease (20). No increase in FGF-BP expression was observed in this model (not shown). This finding corroborates the lack of expression of FGF-BP in human clinical inflammatory bowel disease of different stages (see above). We concluded from these studies that FGF-BP expression is induced during the initiation of malignancy and we hypothesized that this could occur a result of the activation of the Wnt/β-catenin pathway.

Lithium induces endogenous FGF-BP mRNA expression.

To determine if β-catenin is directly involved in the regulation of the FGF-BP gene, we examined whether lithium-induced β-catenin stabilization affects the levels of

endogenous FGF-BP mRNA. Lithium inhibits glycogen synthase kinase-3β (GSK-3β), a negative regulator of β-catenin (21). For the experiments we used MDA-MB468 breast cancer cells, because they express detectable FGF-BP and show intact β-catenin regulation. The MDA-MB468 cells were treated for 16 hours with LiCl and inositol, which prevents IP_3 depletion by LiCl (21). Cells treated with LiCl and inositol increased β-catenin protein levels and showed a 3-fold induction of FGF-BP mRNA as compared to control treatment (NaCl+inositol) (Fig. 3). The NaCl+inositol control showed no significant effect on basal FGF-BP mRNA expression (not shown). Thus, increasing the level of free β-catenin coincides with induction of endogenous FGF-BP mRNA, lending further support to the notion of β-catenin as a regulator of FGF-BP expression.

β-catenin regulates FGF-BP promoter activity.

To investigate whether the FGF-BP gene is a transcriptionally regulated target of β-catenin, we co-transfected a wt β-catenin expression vector with an FGF-BP expression vector containing the −1,060/+62 fragment of the FGF-BP promoter upstream of a luciferase reporter. This 1,060 bp FGF-BP promoter fragment contains numerous consensus transcription factor binding sites that are necessary for the transcriptional activity of the promoter (15). In the CaCo-2 colon cancer cell line, which harbors an APC mutation and expresses endogenous FGF-BP, we found that β-catenin expression induces FGF-BP promoter activity up to 3-fold over basal levels (Fig. 4A). This increase in promoter activity in CaCo-2 cells is comparable to β-catenin induction of the TopFlash promoter, a known β-catenin sensitive promoter containing multimerized TCF sites (not shown).

Because CaCo-2 cells harbor an APC mutation that results in high levels of endogenous β-catenin, we chose the SKBR3 breast cancer cell line as a model for a next series of studies since these cells have no known mutations in the APC/β-catenin signaling pathway and express low basal levels of β-catenin. These cells thus provide a more sensitive system to observe the effects of β-catenin overexpression and dissect the pathway of induction. In these cells, the FGF-BP promoter activity is induced by expression of exogenous β-catenin (Fig. 4B). To determine whether this induction is a β-catenin specific effect, we co-transfected E-cadherin, an adhesion molecule that binds to the internal armadillo repeats of β-catenin and functions as a dominant-negative regulator of β-catenin by preventing its translocation to the nucleus. Co-transfection with E-cadherin reversed β-catenin induction of the FGF-BP promoter (Fig. 4B). We also found that FGF-BP promoter activity of the full-length promoter –1,060/+62 was reduced in isogenic HCT-116 colon cancer cells with somatic cell knock-out of the activated β-catenin allele (14) as compared to promoter activity in wild-type HCT-116 cells (Fig. 4D). Taken together with the results of the β-catenin effects on endogenous levels of FGF-BP mRNA, these data indicate that β-catenin is a transcriptional regulator of the FGF-BP gene.

β-catenin regulatory region in the FGF-BP promoter.

To identify the regions necessary for regulation of the FGF-BP promoter by β-catenin, we transfected SKBR3 cells with 5' deletion constructs of the FGF-BP promoter/reporter constructs (15). β-catenin had a minimal background effect (less than 2-fold) on luciferase activity of the pGL3-basic empty vector (Fig. 4B), similar to non-specific

background effects that we observed previously with this vector (15). Deletion from −1,060 to −118 reduced the β-catenin induced promoter activity by more than 70%. A further deletion to −93 had no effect on the induction of the promoter by β-catenin, but deletion to −77 negated all β-catenin induction of the promoter to background levels of the pGL3 vector (Fig. 4B). The experiments with the FGF-BP promoter/reporter constructs in the HCT-116 knock-out cells which have their activated β-catenin allele deleted (14) showed a significant reduction of constitutive promoter activity of the full length construct. Constitutive activity of the −118/+62 construct, however, was not altered by the deletion of the activated β-catenin allele (Fig. 4D). This finding complements the different inducibility of the activity of these constructs by transfection of exogenous β-catenin in the SKBR3 cells (see Fig. 4B).

We found that the FGF-BP promoter contains two TCF consensus binding sites at −545 and −1,030 and deleted these sites by PCR. Interestingly only deletion of the distal TCF site at −1,030 reduced β-catenin induction of promoter activity whereas deletion of the proximal site at −545 had no significant effect (Fig. 4C). We conclude from these results that β-catenin induction of FGF-BP promoter activity involves regulatory regions in the distal promoter.

Discussion

In this study, we demonstrate that FGF-BP expression is upregulated during early stages of human colon epithelial malignant progression, i.e. during the earliest dysplastic stages associated with a loss of the APC tumor suppressor gene, and is induced in the intestinal adenomas of the B6 ApcMin/+ mouse. We show that FGF-BP expression coincides with the expression of β-catenin in early lesions, i.e. adenomas and already an aberrant crypt focus in the B6 ApcMin/+ mouse, suggesting that FGF-BP lies downstream of the β-catenin signaling cascade. Finally, the studies in cultured cells show that β-catenin activates transcription from the FGF-BP promoter thus providing evidence that this gene is a target of β-catenin.

Our previous analysis of FGF-BP expression in the developing mouse gut had shown that epithelial cells positioned at the bottom of the crypts express FGF-BP and that this expression is lost in cells maturing along the crypt/villus axis (4). More recently, positioning of epithelial cells along the crypt/villous axis and imposition of a crypt precursor phenotype was found associated with a gradient of β-catenin/TCF activity that shows its maximum at the bottom of the crypts and is reduced as cells differentiate during their migration up the crypt (22, 23). It is likely, that the FGF-BP expression that we observed in histologically normal tissues represents staining of sections of the lower third of crypts and hence the region with high β-catenin activity. Also, FGF-BP expression may indicate a very early stage in the transition to dysplasia that is not yet manifest from the H&E staining. Fig. 1A&B shows such an example of dysplastic lesions with surrounding normal mucosa. Interestingly the histologically normal crypts that do not express FGF-BP, show some staining for the protein in sections that transverse the

bottom of the crypt as indicated by the narrow opening of the crypt (compare the two crypts indicated by open arrows in Fig. 1A). With respect to distinct pathologic alterations, the lack of FGF-BP expression in inflammatory human bowel disease and in the rodent animal model equivalent (20) suggest that inflammatory pathways in the colon do not lead to an upregulation of FGF-BP either on their own or through cross-talk with the β-catenin signaling.

FGF-BP is an activator of growth factors in the FGF-family and our studies lend support to the idea that the FGF family plays a role in the development of the early angiogenic phenotype in colon cancer. The induction of the angiogenic phenotype in colon cancer is a multifaceted process requiring the cooperation of numerous factors during the different steps of malignant progression. In a study of levels of FGF-2 and VEGF in serum samples from colon cancer patients, it was suggested that FGF-2 may act as an early inducer of the angiogenic phenotype (24). FGF-BP up-regulation in intestinal adenomas may indeed trigger this by providing the chaperone that can release the immobilized FGF-2. In support of this notion, we found increased angiogenesis coincident with FGF-BP expression in human colon dysplastic lesions. Other factors, such as VEGF, probably cooperate with FGF-2 to maintain the process of angiogenesis throughout the stages of tumor formation. VEGF expression is found in adenomas of the colon, however, unlike our findings with FGF-BP, increased VEGF expression levels are correlated with later stages of the disease and VEGF expression is increased in carcinomas as compared with adenomas as well as in metastatic versus nonmetastatic colon cancer (25).

Not only does FGF-BP appear to be a novel pro-angiogenic target of β-catenin that is upregulated at an early stage of pre-malignant lesions, it seems that its regulation is through areas of the FGF-BP promoter that are not required for basal, growth factor or TPA regulation of this gene in either squamous cell carcinoma or breast cancer cells (see e.g.15, 26). In fact, the regulation by EGF and TPA in these cell types involves an AP-1, a C/EBPβ site and an E-box repressor that are all situated downstream of -118 and that are activated predominantly through the p38/MAPK pathway. Therefore, an unexpected result was the involvement of the region between -1,060 and -118 in the β-catenin regulation of FGF-BP. This also indicates that β-catenin is not activating the promoter via indirect activation of the MAPK signaling pathways. Examination of the 1 kb promoter region between -1,060 and -118 for possible transcription factor recognition sites using Transfac analysis revealed two potential TCF/ LEF sites which are known to be involved in β-catenin regulated gene transcription. Deletion of these sites showed that only the −1,030 site contributes to β-catenin induced promoter activation whereas the site at −545 does not. Interestingly, there is also a perfect consensus site for REL/NFk B present in this region, which is of interest because β-catenin can interact directly with NfkB and might thus contribute in addition to regulation of the gene promoter (27). The second surprise of the FGF-BP gene promoter analysis was that the region between −93 and −77 was also required for full β-catenin induction of the promoter. We have previously demonstrated that this region harbors an SP1 binding site and can bind SP1 specifically. However, this site is not required for growth factor or TPA regulated gene transcription (15). It remains to be determined if this site acts cooperatively with upstream regulatory factors in the β-catenin induction of FGF-BP gene transcription. The Sp1 and Krueppel-

like factors that bind to GC boxes are known to play a role in cell growth and tumor progression. However, their role in early events in colon carcinogenesis has not been defined.

In conclusion, β-catenin, one the most significant oncogenic proteins in colon cancer, has been implicated in several key steps of the path to malignancy. The cell cycle regulatory genes, c-Myc and cyclin-D1 have both been identified as targets of β-catenin. These two proteins were also found overexpressed in intestinal adenomas of the B6 ApcMin/+ mouse. Furthermore, β-catenin activates the matrix metalloproteinase MMP-7, an enzyme that plays a role in invasion and metastasis. Additionally, APC and E-cadherin, two proteins that are closely tied to β-catenin function, are important for induction of apoptosis and cell-cell adhesion, respectively. Our identification of FGF-BP as a direct target of β-catenin transcriptional activation suggests that β-catenin can also play a role in promoting the switch to the angiogenic phenotype observed early in the malignant progression of colon cancer.

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Figure Legends

Fig. 1. FGF-BP1 expression in human colorectal samples.

A: Immunohistochemistry using a polyclonal antibody against FGF-BP1 shows FGF-BP1 protein expression in colonic crypts. Normal colon crypts (open arrows) and a dysplastic crypt (closed arrow) are indicated.

B: Magnification of the region indicated in panel A by the dotted frame.

C: *In situ* hybridization for FGF-BP1 mRNA of a colon polyp with dysplastic crypts.

D: Frequency of FGF-BP1 protein expression using 161 different biopsies from patients with normal epithelium and dysplastic lesions. ***, $p<0.0001$, chi-square.

Fig. 2. FGF-BP and β-catenin expression in B6 ApcMin/+ mouse samples. Dysplastic lesions and adjacent normal mucosa is shown. In E,F,G,H (6x mag.) the asterisk (*) indicates the identical area in each slide for orientation purposes.

A, E: H&E stained section. Dysplastic lesions appear in dark blue.

B, F: Staining for β-catenin protein.

C, G: *In situ* hybridization for FGF-BP mRNA. Antisense probe. Expression of FGF-BP is confined to the dysplastic region.

D,H: FGF-BP sense probe as a negative control.

I,J: Staining for FGF-BP protein (I) and β-catenin protein (J) in a colon aberrant crypt focus that is surrounded by normal mucosa.

K: Frequency of FGF-BP expression in normal mucosa and polyps. **, $p<0.001$, chi-square.

Fig. 3. Induction of FGF-BP mRNA by lithium chloride in MDA-MB468 cells.

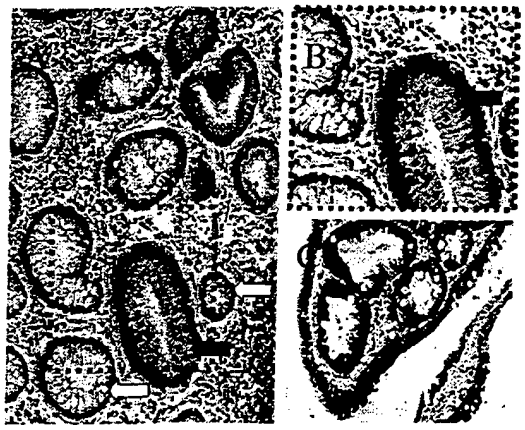
A: Northern blot for FGF-BP mRNA and western blot for β−catenin. α-tubulin is a loading control. Treatment was for 16 h. Duplicate samples were loaded. The experiment was repeated 3 times.

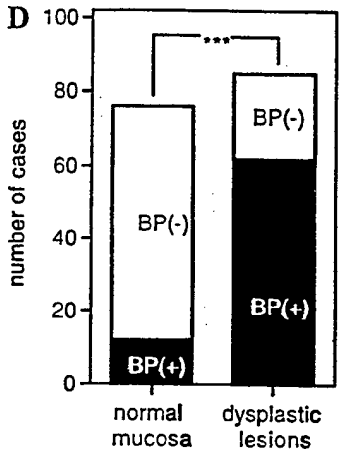
B: Levels of FGF-BP mRNA were quantified by Phosphoimaging and corrected for loading by GAPDH mRNA levels. **, $p<0.01$ relative to NaCl treatment.

Fig. 4. Effect of β-catenin on FGF-BP promoter activity. NEEDS WORK

CaCo-2 (A), SKBR3 (B,C) and HCT116 (D) cells were used. Different promoter/reporter constructs were transiently transfected as indicated plus either pcDNA or β-catenin ± E-cadherin and luciferase activity was measured after 18 hours. E-cadherin was included with the −1,060/+62 construct. Effect of the deletion of the consensus TCF site (5'-A/T A/T CAAAG-3' on β-catenin induction of FGF-BP promoter activity. Means +/- S.E. from three different experiments (A,B) and a representative experiment from two independent repeats (C) are shown. *, $p<0.05$ relative to empty vector; #, $p<0.05$ relative to −1,060/+62.

D: Effect of somatic cell knock-out of the mutated and activated β-catenin allele in HCT116 cells. The FGF-BP promoter/reporter constructs were transiently transfected (Fugene, Roche, 48 hours) into the wild-type (wt) HCT116 colon cancer cell line that contains an activated β-catenin allele or into an isogenic knock-out cell line (ko) in which the activated β-catenin allele was deleted (14). A representative experiment from two independent repeats with triplicate measurements each is shown. *, $p<0.05$ relative to wt.





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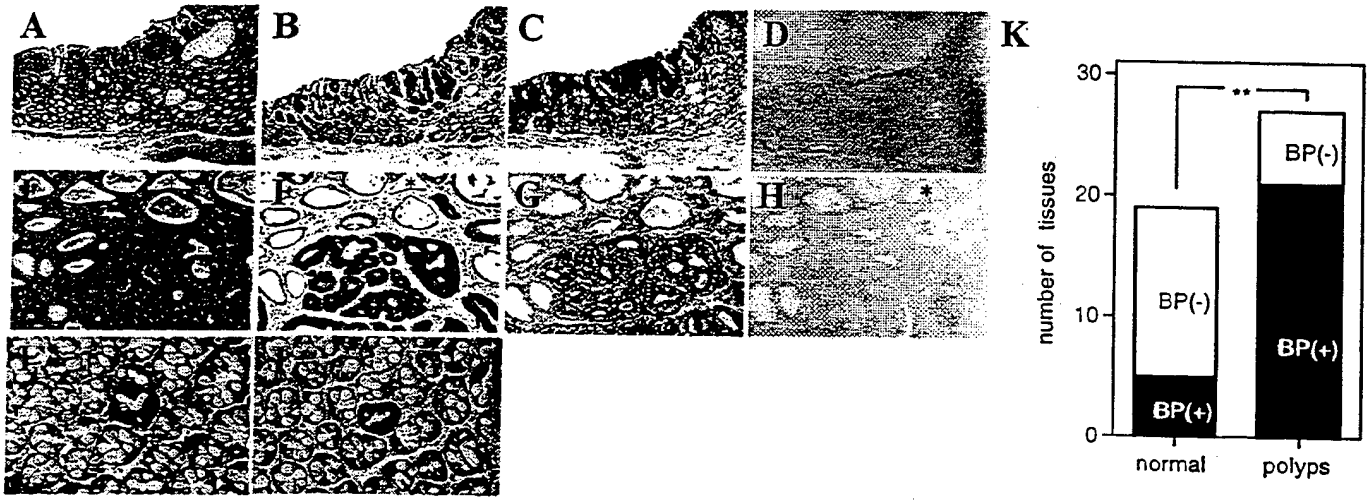

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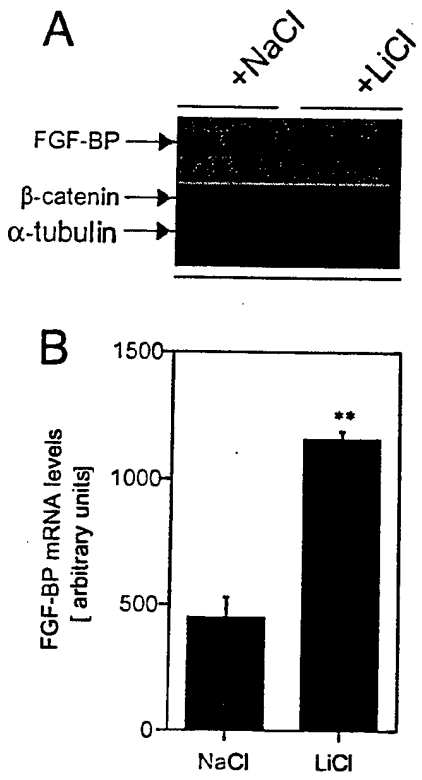

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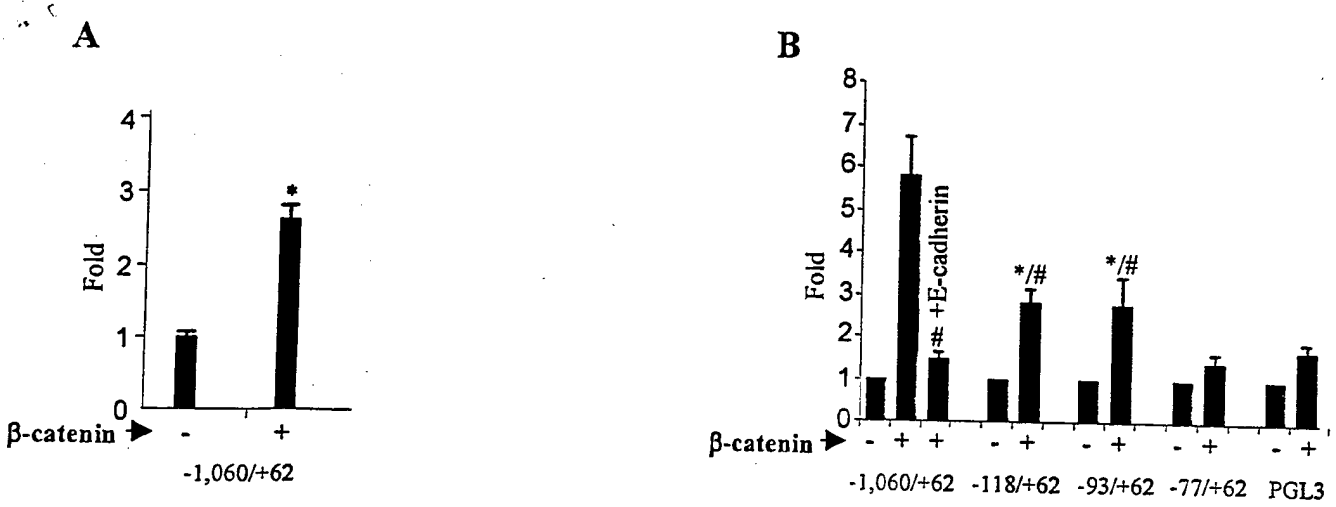
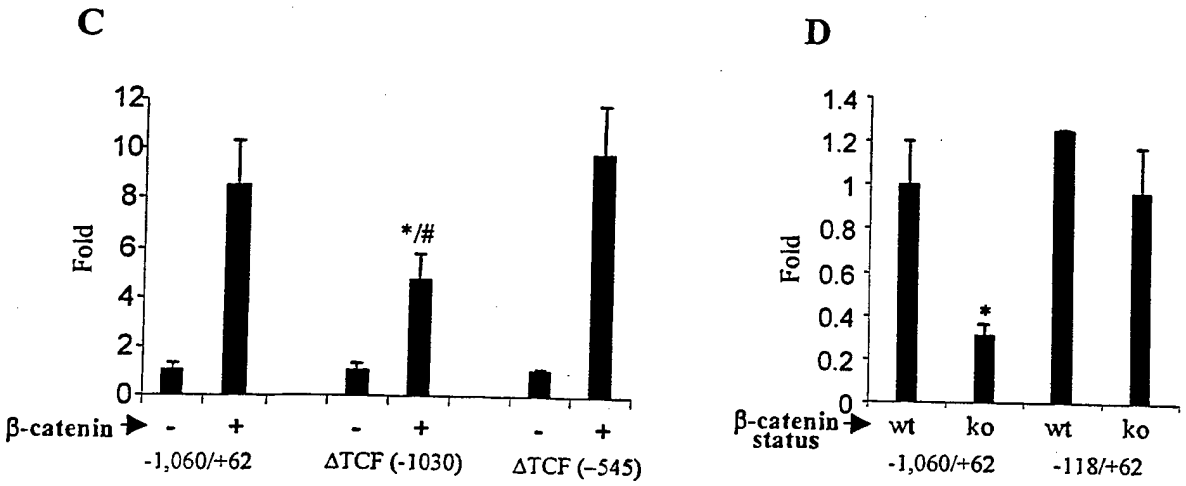


FGF-BP promoter/reporter constructs



FGF-BP promoter/reporter constructs

Ray et al. Figure 4